

Role Bending: Complex Relationships Between Viruses, Hosts, and Vectors Related to Citrus Leprosis, an Emerging Disease

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ABSTRACT

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Citrus leprosis complex is an emerging disease in the Americas, associated with two unrelated taxa of viruses distributed in South, Central, and North America. The cytoplasmic viruses are *Citrus leprosis virus C* (CiLV-C), *Citrus leprosis virus C2* (CiLV-C2), and *Hibiscus green spot virus 2*, and the nuclear viruses are *Citrus leprosis virus N* (CiLV-N) and *Citrus necrotic spot virus*. These viruses cause local lesion infections in all known hosts, with no natural systemic host identified to date. All leprosis viruses were believed to be transmitted by one species of mite, *Brevipalpus phoenicis*. However, mites collected from CiLV-C and

CiLV-N infected citrus groves in Mexico were identified as *B. yothersi* and *B. californicus* sensu lato, respectively, and only *B. yothersi* was detected from CiLV-C2 and CiLV-N mixed infections in the Orinoco regions of Colombia. Phylogenetic analysis of the helicase, RNA-dependent RNA polymerase 2 domains and p24 gene amino acid sequences of cytoplasmic leprosis viruses showed a close relationship with recently deposited mosquito-borne negevirus sequences. Here, we present evidence that both cytoplasmic and nuclear viruses seem to replicate in viruliferous *Brevipalpus* species. The possible replication in the mite vector and the close relationship with mosquito borne negevirus are consistent with the concept that members of the genus *Cilevirus* and *Higrevirus* originated in mites and citrus may play the role of mite virus vector.

Additional keywords: *Dichorhavirus*, flat mite, virus replication.

Citrus leprosis, one of the most important emerging citrus diseases in South and Central America, causes severe defoliation, girdled limbs, premature fruit drop, death of twigs, and reduction in both fruit quality and yield, as well as tree death within 3 to 5 years. The disease is not present in the United States, although leprosis was first reported in Florida during 1860s (Knorr 1968) and resulted in serious impacts on citrus production. After 1926, the incidence of leprosis in Florida began to decline. Leprosis was present only in isolated areas on the east coast of Florida in the 1950s (Knorr 1968), and surveys since 1968 have not resulted in any findings in Florida (Childers et al. 2003a). The first report of citrus leprosis in Brazil was in 1931, on sweet orange trees growing in Sorocaba, São Paulo (Bitancourt 1934), with subsequent findings in Argentina (Bitancourt 1940). Today cytoplasmic leprosis is present throughout most of Brazil, most prominently in the south and southeastern states including the citrus-producing regions of São Paulo (Bastianel et al. 2010; Muller et al. 2005; Rodrigues et al. 2003; Salva and Massari 1995). In addition, nuclear leprosis was first reported from Brazil (states of São Paulo,

Minas Gerais, and Rio Grande do Sul) followed by Panama, Mexico and most recently Colombia (Bastianel et al. 2010; Rodrigues et al. 2003; Roy et al. 2013b, 2014). Leprosis has been reported in Colombia along the foothills of the Eastern Mountains of the Orinoco region (León et al. 2006a; Roy et al. 2013a, 2014). In 2005, *Citrus leprosis virus C* (CiLV-C) was first reported from Tabasco and Chiapas, two states in southern Mexico (Izquierdo-Castillo et al. 2011), and in 2012 *Citrus leprosis virus N* (CiLV-N) was reported from Querétaro state, in central Mexico (Roy et al. 2013b) (Fig. 1).

The disease symptoms associated with leprosis are caused by two taxonomically distinct classes of viruses, positive-sense RNA viruses from the genera *Cilevirus* and *Higrevirus*, and negative-sense RNA viruses from the newly proposed genus *Dichorhavirus*. CiLV-C and CiLV-C2 are members of the genus *Cilevirus*, *Hibiscus green spot virus 2* (HGSV-2) is the type member of the genus *Higrevirus*, and CiLV-N and *Citrus necrotic spot virus* (CiNSV) are members of the genus *Dichorhavirus*, a proposed genus in the family *Rhabdoviridae* (Dietzgen et al. 2014). Leprosis-associated viruses are present in South, Central, and North America and are either transmitted by or associated with *Brevipalpus* spp., although no confirmation of mite transmission has been recorded for CiLV-N, CiNSV, and HGSV-2 (Cruz-Jaramillo et al. 2014; Melzer et al. 2012; Roy et al. 2015).

Brevipalpus mites (Tetranychoidae: Tenuipalpidae) are present throughout the world (Beard et al. 2014, 2015). These mites are

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polyphagous in nature, making them very difficult to control. Research on *Brevipalpus* mites was somewhat neglected until their capacity to transmit pathogenic viruses was demonstrated (Childers and Derrick 2003; Kitajima et al. 2003). The genus *Brevipalpus*, with more than 300 nominal species, is the second largest genus in the family Tenuipalpidae (Beard et al. 2013; Mesa et al. 2009). Three species of *Brevipalpus* have been implicated in the transmission of several viruses: *B. phoenicis* sensu lato (s.l.) (Geijskes), *B. californicus* s.l. Banks, and *B. obovatus* Donnadieu (Kitajima et al. 2001, 2003; Kitajima and Alberti 2014; Maeda et al. 1998; Rodrigues et al. 2008; Rodrigues and Childers 2013). The identity of the mite species that serve as vectors of citrus leprosis viruses are yet to be determined, as the taxonomic status of these individual species is currently uncertain and voucher specimens are rarely available for study. Mites historically identified as *B. californicus*, *B. obovatus*, and *B. phoenicis* are widespread in South America, including Brazil, where they have been recorded from at least 234 plant species from 72 families (Childers et al. 2003b; Miranda et al. 2007). Frezzi (1940) demonstrated an association between *Brevipalpus* mites and citrus leprosis in Argentina (“lepra explosiva”), and likewise, Musumecchi and Rossetti (1963) made the first association between *B. phoenicis* s.l. mites and citrus leprosis in Brazil through field observations and transmission of the disease using mites under experimental conditions. Mites associated with citrus leprosis symptoms in Florida, including voucher specimens, were confirmed as *B. californicus* s.l., suggesting this species group to have been the most likely vector of leprosis in Florida. Although most of these mounted specimens were identified as *B. californicus* s.l., some of specimens also were identified as *B. yothersi* Baker, a former synonym of *B. phoenicis* s.l. (Beard et al. 2014, 2015).

In 2009, *Citrus volkameriana* trees growing in Waimanalo (Oahu, Hawaii), displaying local lesion symptoms similar to citrus leprosis were observed. Short, bacilliform virus-like particles similar to those of CiLV-C were present in the cytoplasm of symptomatic, but not asymptomatic leaf tissues. However, molecular characterization revealed that a novel, tripartite ssRNA virus distantly related to CiLV-C was present in symptomatic tissues (Melzer et al. 2012). This virus was also found in *Hibiscus arnottianus* trees displaying large, green ringspots in senescent

leaves. In 2013, another *Cilevirus* closely related to CiLV-C2 (Tables 1 and 2, described as CiLV-C2H) was discovered in *H. rosa-sinensis* growing in Honolulu, HI (Melzer et al. 2013). Symptomatic plants displayed green spots and ringspots in senescing leaves, and could not be detected by a CiLV-C2-specific reverse transcription-polymerase chain reaction (RT-PCR) assay (Roy et al. 2013a). Symptomatic ornamental hibiscus plants have been subsequently observed at locations on the islands of Oahu, Kauai, Maui, and Hawaii (M. Melzer, unpublished data), suggesting the virus is widespread in Hawaii. The symptoms observed in Hawaii resemble the ringspot symptoms observed in Brazilian-grown hibiscus infected with the cytoplasmic virus designated as *Hibiscus green spot virus* (HGSV) (Kitajima et al. 2003). It is not known if the cilevirus identified in hibiscus is able to infect and induce symptoms in citrus, nor whether it is vectored by *B. yothersi*, the previously identified vector (*B. phoenicis*) of CiLV-C2 (Roy et al. 2013a).

Currently there are no known systemic plant hosts for these viruses. Sweet orange (*C. × sinensis*), grapefruit (*C. × paradisi*), and mandarin (*C. reticulata*) are susceptible to CiLV-C infection, but lemons (*C. × limon*) are immune (Bastianel et al. 2010). *Swinglea glutinosa*, a perennial ornamental, and *Commelina benghalensis*, a weed plant, were found to be naturally infected with CiLV-C in Meta, Colombia (León et al. 2008) and São Paulo, Brazil (Nunes et al. 2012a), respectively. Experimentally, *Chenopodium quinoa*, *C. amaranticolor*, *Gomphrena globosa*, *H. rosa-sinensis*, *Malvaviscus arboreus*, *Grevillea robusta*, *Phaseolus vulgaris*, and *Solanum*

TABLE 1. Percent nucleotide (above diagonal *) and amino acid (below diagonal *) identities of RNA1 genomes of *Citrus leprosis virus C* (CiLV-C), CiLV-C2, and hibiscus infecting *Cilevirus* sp. (CiLV-C2H) associated with leprosis-like symptoms^a

SI number	Viruses	RNA1								
		ORF1 (p285)			ORF2 (p29)			Complete genome		
		1	2	3	1	2	3	1	2	3
1	CiLV-C	*	59	59	*	46	47	*	58	58
2	CiLV-C2	58	*	85	34	*	92	ND	*	86
3	CiLV-C2H	58	94	*	34	94	*	ND	ND	*

^a ND indicates data not determined.

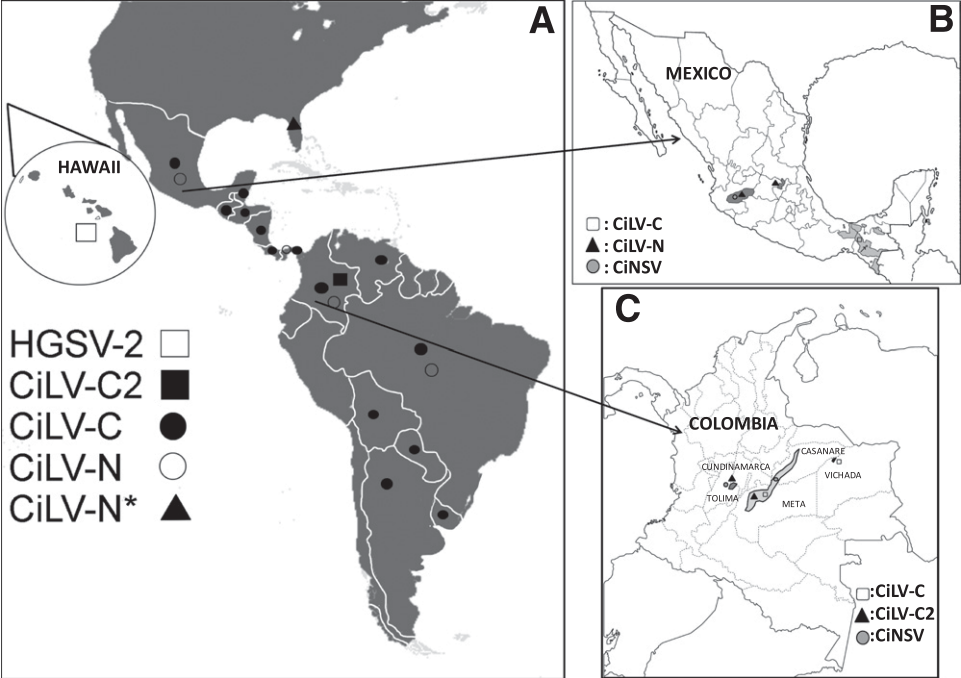


Fig. 1. Geographical distribution of viruses associated with citrus leprosis disease. A, Detailed emergence and distribution of cytoplasmic and nuclear citrus leprosis viruses in B, Mexico and C, Colombia. Asterisk (*) represents the presence of *Citrus leprosis virus N* (CiLV-N) in Florida prior to 1968.

violaeifolium have been reported as hosts for CiLV-C by different authors (Colariccio et al. 1995; Garita et al. 2013; Groot et al. 2006; Nunes et al. 2012a; Rodrigues et al. 2005). In an expanded experimental host range study of CiLV-C, 140 plant species from 43 families were tested, and 40 species belonging to 18 families produced either localized chlorotic and/or necrotic lesions on leaves identified as positive for CiLV-C (Garita et al. 2014). CiLV-C2 has been recorded from four sweet orange cultivars ('Valencia', 'Delta Valencia', 'Rhode Red Valencia', and 'Cara Cara Navel') (Roy et al. 2013a). *C. volkameriana* and *H. arnottianus* have been reported as hosts of HGSV-2 (Melzer et al. 2012). Field infection of seven citrus species: grapefruit, lemon, lime (*C. × aurantifolia*), mandarin, sour orange (*C. aurantium*), sweet lime (*C. limetta*), and sweet orange by CiLV-N, and four citrus species: grapefruit, sour orange, sweet orange, and tangerine by CiNSV have been reported in states of Querétaro and Jalisco, Mexico, respectively (Cruz-Jaramillo et al. 2014; Roy et al. 2015).

In this study, novel hosts are reported for CiLV-C2, CiLV-N, and HGSV-2. A careful examination of mites from newly emerging outbreaks identified the vector of CiLV-C as *B. yothersi* and the vector of CiLV-N as *B. californicus* s.l. In addition, data that supports the replication of CiLV-C, -C2, and -N in mites and the genetic relationship of cileviruses with insect viruses suggests that the traditional views of plants as hosts and insects as vectors may not apply to viruses associated with citrus leprosis.

MATERIALS AND METHODS

Sources of leprosis-associated viruses from different countries. Citrus orchards in Meta and Casanare states in Colombia infected with CiLV-C2 or CiLV-N or both viruses were revisited during 2013–14 and leprosis-affected Valencia sweet orange leaves, twigs, and fruits were collected. During the survey leprosis-like symptoms were also observed in *S. glutinosa*, *Dieffenbachia* sp., and *Hibiscus* sp. in Meta state of Colombia. To confirm the presence of leprosis-associated viruses, both symptomatic and nonsymptomatic samples were collected from citrus groves and surrounding areas. To establish the distribution of CiLV-C and CiLV-N in Mexico, a survey was conducted in June 2014. CiLV-C and -N suspect samples and associated *Brevipalpus* mites from Tabasco and Querétaro states of Mexico, respectively, were collected. Samples were collected from eight different citrus species: grapefruit, lemon, key lime, mandarin, Persian lime, sour orange, sweet lime, and sweet orange. Healthy leaf samples, leprosis-affected leaf samples, and their associated mites were examined to identify the viruses and mites as described below.

In Kula on the island of Maui, Hawaii, a citrus farm growing primarily mandarin, sweet orange, and Persian lime was visually inspected for leaf blotch symptoms reported by the citrus grower. Approximately 60 trees of each variety were inspected. Symptomatic and asymptomatic leaf and fruit tissue were collected from the field and transported in double containment on ice to the laboratory and stored at 4°C. In addition, a small hau tree (*H. tiliaceus*) growing adjacent to *H. arnottianus* plants infected with HGSV-2 on the University of Hawaii at Manoa campus displayed green and red leaf blotch symptoms on senescing leaves, which were similar in

appearance to the symptoms displayed by *H. arnottianus* infected with HGSV-2. Symptomatic leaves were collected and stored at 4°C for RT-PCR detection assay.

RNA extraction and RT-PCR. Approximately 100 mg of leaf tissue (equivalent to 6 to 12 lesions) or fruit peel of healthy and leprosis-affected citrus, *S. glutinosa*, *Dieffenbachia* sp., and *Hibiscus* sp. was frozen in liquid nitrogen and pulverized with a mortar and pestle. Total RNA was then isolated using a Qiagen RNeasy Mini kit (Valencia, CA) following the manufacturer's instructions. Total RNA was eluted in 60 µl of nuclease-free water. For some samples total RNA was extracted from single or overlapped lesions using the same approach. RNA titers were quantified by spectrophotometry, and equal amounts of RNA were used as templates for RT-PCR. Primer pairs specific to CiLV-C (Locali et al. 2003), CiLV-C2 (Roy et al. 2013a), CiLV-N (Roy et al. 2015), and HGSV-2 (Melzer et al. 2012) were used to detect these viruses from leprosis-affected tissues collected from Colombia, Hawaii, and Mexico following the published RT-PCR protocol (Roy et al. 2013a). Moreover, primers specific for cileviruses that infect hibiscus (Melzer et al. 2013) also were utilized to detect viruses related to CiLV-C2 in these samples. The PCR amplicons were resolved in 1% agarose gels and stained with GelRed (Biotium, Hayward, CA) and visualized under UV light, and then cloned with the TOPO TA cloning vector (Life Technologies, Carlsbad, CA) following the manufacturer's protocol. The clones were bidirectionally sequenced.

Identification of *Brevipalpus* spp. using low temperature scanning electron microscopy (LT-SEM). Slide-mounted specimens of *Brevipalpus* spp. were collected from the Americas and examined at ×1,000 magnification using a DIC and phase contrast Zeiss Axioscope microscope. As both morphological and molecular evidence strongly suggests that the taxa *B. phoenicis* and *B. californicus* actually comprise several cryptic species (Beard et al. 2013; 2015; Navia et al. 2013; Rodrigues et al. 2004), our *Brevipalpus* spp. identifications were made utilizing multiple distinguishing characters (Table 3, Supplementary Figs. 1 and 2), including cuticular microplates as outlined in Beard et al. (2015). Due to the recent taxonomic resolution within these species groups, specimens previously identified as *B. phoenicis* or *B. californicus* are referred to here as *B. phoenicis* s.l. and *B. californicus* s.l. unless vouchers were examined and a current species identification could be made. The true *B. phoenicis* sensu stricto, while recorded on *Citrus* spp. hosts, has not yet been associated with citrus leprosis (Beard et al. 2015). Specimens in 70% ethanol from Brazil (5 samples), Colombia (3 samples), and Mexico (21 samples) were also used for LT-SEM studies, utilizing the technique outlined by Bolton et al. (2014). Images were captured using a 4pi Analysis System (Durham, NC). Because the specimens were not tightly secured to the adhesive tabs, it was often possible to remove the specimens from the LT-SEM and turn them over to the view ventral side; thus, both dorsal and ventral images were taken of individual mites.

Mite transmission. Suspected nonviruliferous *Brevipalpus* mites were collected from a leprosis-free citrus grove and an established leprosis virus-free laboratory mite colony in Colombia following previously described protocols (Nunes et al. 2012b; Roy et al. 2013a).

TABLE 2. Percent nucleotide (above diagonal*) and amino acid (below diagonal*) identities of RNA2 genomes of *Citrus leprosis virus C* (CiLV-C), CiLV-C2, and hibiscus infecting *Cilevirus* sp. (CiLV-C2H) associated with leprosis-like symptoms^a

SI number	Viruses	RNA2																	
		ORF1(p15)			ORF2 (p7)			ORF3 (p61)			ORF4 (p32)			ORF5 (p24)			Complete genome		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	CiLV-C	*	39	41	*	ND	ND	*	47	46	*	55	52	*	60	61	*	47	47
2	CiLV-C2	15	*	79	ND	*	88	30	*	83	50	*	83	60	*	88	ND	*	78
3	CiLV-C2H	17	77	*	ND	50	*	31	85	*	49	91	*	60	96	*	ND	ND	*

^a ND indicates data not determined.

For transmission studies, 80 to 100 healthy mites were fed on *C. sinensis* and *S. glutinosa* leaves with leprosis lesions. After a 24 h acquisition access period (AAP), approximately 50 mites were collected, submerged in 90% ethanol, and later assayed for CiLVs. The remaining mites were placed on virus-free sweet orange and *S. glutinosa* seedlings to continue the transmission experiment in order to confirm Koch's postulates following the protocol described by Roy et al. (2013a). The experiment was repeated twice using four replications. To detect the presence or absence of leprosis viruses in suspected viruliferous and nonviruliferous *Brevipalpus* sp., approximately 15 to 20 mites from each group were collected and tested by leprosis virus specific RT-PCR as described by Roy et al. (2015).

Detection of leprosis viruses in *Brevipalpus* sp. vectors by RT-PCR. Nonviruliferous *Brevipalpus* mites were collected from a citrus leprosis-free orchard and from virus-free mite colonies in La Libertad, Meta, Colombia. Two suspected viruliferous *Brevipalpus* mite samples were collected from a leprosis-affected orchard in La Libertad and a third mite sample was collected from a leprosis virus transmission study. To detect leprosis viruses inside the mites, after the 24 h AAP, approximately 40 to 50 mites were frozen in liquid nitrogen and crushed in 2-ml Eppendorf tubes using a small plastic pestle. Total mite RNA was extracted using the Qiagen RNeasy Fibrous Tissue Mini kit (Valencia, CA) following the manufacturer's instructions, with final elution using 15 to 20 µl of elution buffer. RNA yield and quality was measured with a NanoDrop ND-1000 Spectrophotometer (Wilmington, DE) and the RNA was stored at -20°C for subsequent analysis. A mixture of leprosis-associated virus-specific reverse strand primers (2 µM) (Locali et al. 2003; Melzer et al. 2012, 2013; Roy et al. 2013a, 2014b) was added separately to the total RNA of nonviruliferous and viruliferous mites and cDNA synthesis (total volume 25 µl) followed the manufacturer's protocol (Invitrogen, Carlsbad, CA). PCR was performed and amplicons were resolved by gel electrophoresis, purified, cloned, and bidirectionally sequenced using the previously described protocol (Roy et al. 2013a).

Detection of leprosis virus replication within *Brevipalpus* spp. The modified two-step RT-PCR protocol described above (Kubo et al. 2011, Roy et al. 2013a) also was performed to detect replication intermediates of leprosis viruses (CiLV-C, CiLV-C2, and CiLV-N) in *Brevipalpus* mites. Negative-strand RNA specific cDNA from CiLV-C and CiLV-C2 was synthesized using a forward primer during cDNA synthesis, and positive-strand specific cDNA from CiLV-N was synthesized with the reverse primer that was utilized for RT-PCR detection assays of leprosis-associated viruses (Supplementary Table 1). cDNA was purified using the Qiagen PCR purification kit and PCR was performed following the previously described protocol (Roy et al. 2013a) with leprosis virus species specific forward and reverse primers. To confirm the

presence of CiLV-C, -C2, and -N in *Brevipalpus* mites, the same RNA extracts were used as templates for traditional cDNA synthesis utilizing the reverse primer of the encapsidated strand of each virus.

Phylogenetic analysis. Phylogenies were constructed for the helicase and RNA-dependent RNA polymerase (RdRp), and a hypothetical (p24) protein using deduced amino acid sequences obtained from NCBI GenBank. The helicase and replicase domains of the RdRp2 were extracted using the SMART domain program (<http://smart.embl-heidelberg.de>). p24 sequences were used to search the NCBI nr database and the resulting blast hits were used in the construction of the phylogenetic tree. In some cases sequences were not annotated as a p24 gene, but relevant sequences based on e-values were included in the trees. Amino acid sequences were aligned using Clustal X software. Phylogenies were constructed with the neighbor-joining and maximum parsimony methods in MEGA 6 (Tamura et al. 2013) with 1,000 bootstrap replications. Branches with bootstrap values greater than 70 were retained.

RESULTS

Natural infection of rutaceous and nonrutaceous plants by leprosis-related viruses. Surveys were conducted in Colombia, Hawaii, and Mexico to determine the distribution of the cytoplasmic and nuclear citrus leprosis-associated viruses. During the survey in Colombia, leprosis-like symptoms were observed in *Dieffenbachia* sp. and *S. glutinosa*. The symptoms observed in *S. glutinosa* were distinct from the symptoms noted in *Dieffenbachia* sp. The chlorotic lesions with 'nail head' necrotic centers surrounded by broad yellow haloes observed on *S. glutinosa* (Fig. 2A) were similar to the symptoms produced by CiLV-C on *S. glutinosa* (Leon et al. 2008) and the symptoms of CiLV-C and -C2 observed on different sweet orange cultivars (Roy et al. 2013a). The lesions observed on *Dieffenbachia* sp. were smaller in size, dark brown in color, and without chlorotic haloes (Fig. 2B). Small light brown lesions also were observed, often coalesced in advanced stages of infection (data not shown). No symptoms were observed on twigs of *S. glutinosa* or stems of *Dieffenbachia*. Many green ring lesions with internal chlorotic spots were observed in a senescing *H. rosa-sinensis* leaf in Meta, Colombia (Fig. 2D). *H. rosa-sinensis* showed chlorotic spots but not senescence, when *Brevipalpus* mites infected with CiLV-C were used for experimental inoculation (Nunes et al. 2012b). Persian lime leaves with distinct necrotic lesions with yellow haloes (Fig. 2C) were collected from Querétaro state in Mexico. The necrotic lesions were smaller in size (3 to 5 mm) than typical leprosis symptoms produced by cytoplasmic leprosis viruses (6 to 12 mm). Coalescence of necrotic lesions was not observed.

TABLE 3. Taxonomic criteria for the classification of three *Brevipalpus* species associated with citrus leprosis disease complex

Taxonomic characters	<i>B. californicus</i> s.l. ^a	<i>B. yothersi</i>	<i>B. phoenicis</i> s.l.
Number solenidia on tarsus II	2	2	2
Dorsal opisthosomal setae <i>f2</i>	Present	Absent	Absent
Dorsal cuticle pattern between setae <i>e1</i> and <i>h1</i>	*	V-shaped folds	Transverse folds
Dorsal cuticle pattern laterad setae <i>e1</i>	*	Regular reticulate cells	Large rounded cells
Cuticle pattern on ventral plate	*	Separate warts	Transverse bands
Cuticle pattern on genital plate	*	Large warts/cells	Large warts/cells
Spermatheca	*	Long fine duct ending in oval vesicle with thick distal stipe	Thick duct ending in small distal bulb
Seta on palp femurogenou	*	Narrow, tapered, barbed	Broad, flat, barbed
Cuticular microplate pattern	*	Separate rounded plates with series of parallel ridges dorsally	Separate irregularly shaped plates with irregular multidirectional ridges dorsally

^a Asterisk (*) indicates that *B. californicus* sensu lato is an unresolved species complex, and for this reason each of these characters is highly variable (Beard et al. 2013).

In Hawaii, nearly 100% of the visually inspected mandarin and navel orange trees displayed chlorotic green spots surrounded by yellow haloes (Fig. 3A and C). In addition, immature fruit from some navel orange also displayed small (3 to 4 mm) chlorotic lesions (Fig. 3B). These symptoms differed from symptoms normally associated with leprosis, lacking brown necrotic spots and necrotic rings. No symptoms were observed in any of the inspected Persian lime trees. Leaves of *H. tiliaceus* displayed symptoms of senescence with patches of lesions with distinct green borders (Fig. 3D). *Brevipalpus* mites were observed on all tissues (from Colombia, Hawaii, and Mexico). No *Brevipalpus* mites were observed on the symptomatic *H. tiliaceus* leaf brought back to the laboratory, although these mites have been occasionally observed on leaves subsequently collected from this same tree.

Molecular identification of leprosis-related viruses. RT-PCR assays specific for cytoplasmic and nuclear leprosis viruses were utilized to determine the presence of single or mixed infections of leprosis-associated viruses in rutaceous (mandarin and navel sweet orange and *S. glutinosa*) and nonrutaceous (*Dieffenbachia* sp. and *H. rosa-sinensis*) plants in Colombia, Hawaii, and Mexico. CiLV-C2 species specific primers produced amplicons from *Dieffenbachia* sp., *H. rosa-sinensis*, and *S. glutinosa* samples with symptoms of leprosis obtained from Colombia. CiLV-C primers did not produce amplicons when the same RNA templates were used in RT-PCR. CiLV-N specific RT-PCR confirmed the coinfection of CiLV-N and CiLV-C2 in *Dieffenbachia* sp. and *S. glutinosa*, but CiLV-N was not detected in *H. rosa-sinensis* plants infected with CiLV-C2. RT-PCR assays for other leprosis-associated viruses did

not detect either citrus infecting HGSV-2 or hibiscus infecting *Cilevirus* in Colombia. The CiLV-N and CiLV-C2 amplicons obtained from *Dieffenbachia* sp. and *S. glutinosa* and the CiLV-C2 amplicon from *H. rosa-sinensis* shared 98 to 99% sequence identity with isolates M2345 of CiLV-N and L147V1 of CiLV-C2, respectively. To test if individual lesions represented single infections within a tree with both viruses, four well separated and one coalesced lesion (consisting of merged single lesions) were collected from three leaves taken from four naturally doubly infected plants from Meta and Casanare and one plant derived by experimental transmission (Fig. 4). RT-PCR from both single and multiple lesions detected both CiLV-C2 and -N in all of these samples, but no CiLV-C amplicon was ever detected.

Only CiLV-N species specific primers produced amplicons from symptomatic Persian lime leaf samples collected from Querétaro, Mexico. None of the symptomatic tissues collected from leprosis-infected areas of Tabasco and Querétaro state of Mexico produced amplicons in RT-PCR tests with HGSV-2 or hibiscus-infecting cilevirus specific primers. However, both CiLV-C and CiLV-N specific amplicons were obtained from symptomatic sweet orange samples collected from Tabasco, Mexico, where the incidence of CiLV-C was predominant. CiLV-N and CiLV-C amplicons obtained from infections in Persian lime and sweet orange shared 98 to 99% nucleotide sequence identity with Mexican CiLV-N isolate M2345 (KF209275), strain Jal-1 of CiNSV (KF198064), and Brazilian CiLV-C isolate Cordeiropolis (DQ352194) sequences in GenBank.

In Hawaii, amplification products of the expected size for HGSV-2 were produced from all symptomatic mandarin and navel orange leaf and fruit tissues tested. Amplification products were not observed from either Persian lime leaves or asymptomatic sweet orange fruit rind samples (data not shown). Amplification products from mandarin, sweet orange, and hau (*H. tiliaceus*) samples were sequenced to identify the presence of HGSV-2. All sequences were >97% identical to the HGSV-2 sequence in GenBank (HQ852052).

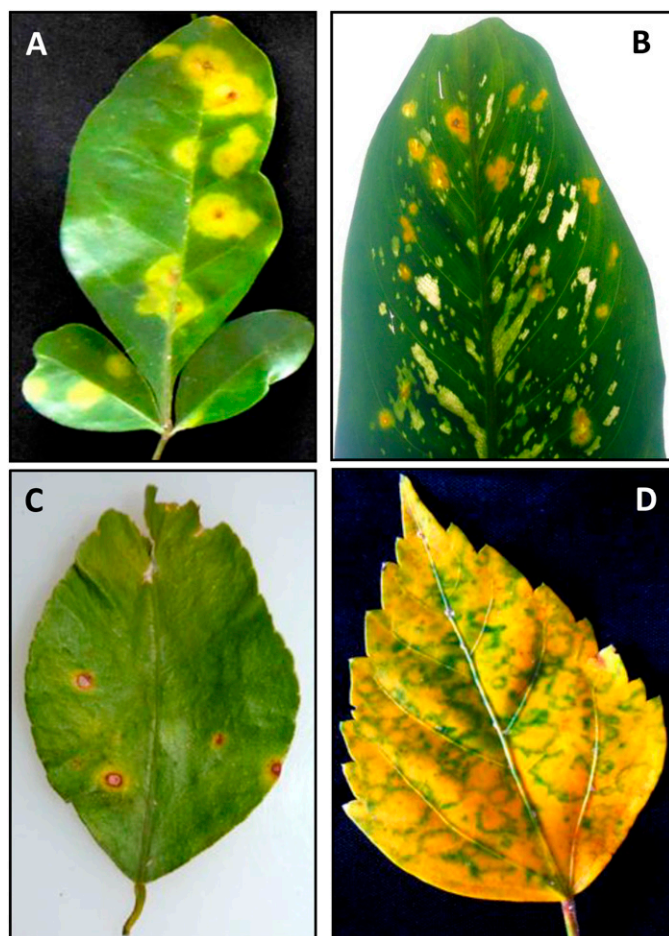


Fig. 2. Natural *Citrus leprosis virus* C2 (CiLV-C2) and CiLV-N mixed infection of **A**, *Swinglea glutinosa*, and **B**, *Dieffenbachia* sp. in Colombia and single infection of **C**, Persian lime (*Citrus latifolia*), and **D**, *Hibiscus rosa-sinensis* by CiLV-N and CiLV-C2 observed in Mexico and Colombia, respectively.

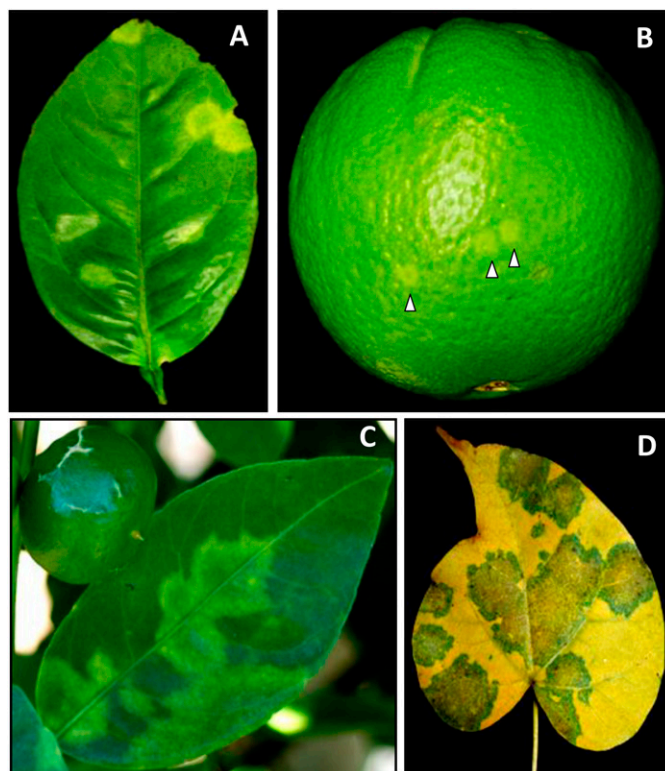


Fig. 3. Natural hosts of *Hibiscus green spot virus* 2 (HGSV-2) include navel sweet orange (*Citrus sinensis*) **A**, leaf, and **B**, fruit. Arrowheads indicate three lesions on immature fruit. **C**, Mandarin (*C. reticulata*) leaf and **D**, hau (*Hibiscus tiliaceus*) leaf infected by HGSV-2.

None of the symptomatic Hawaiian samples produced amplicons with either CiLV-C, -C2, or -N specific primers.

Diversity of viruses associated with symptoms of citrus leprosis. The leprosis related positive-sense viruses have either bipartite (CiLV-C and -C2) or tripartite (HGSV-2) genome structures (Fig. 5) and were observed only in the cytoplasm of infected leaf tissues. Leprosis related cileviruses are very distinct from each other (Tables 1 and 2), even though they produce similar leprosis-like symptoms on different citrus species. CiLV-C shared 62 to 77% and 32 to 40% (helicase<RdRP<methyltransferase) sequence identity with CiLV-C2 and HGSV-2, respectively. In addition to domains of the replicase polyprotein, p24, a probable integral membrane protein of the virion encoded by RNA2 of CiLV-C and -C2, shared 26% nucleotide identity with a similar protein sequence (p23) present in the RNA3 genome of HGSV-2. Further analyses of the p24 or p23 proteins present in these closely related

cytoplasmic *Brevipalpus* mite transmitted or associated plant viruses identified three conserved domains: A (DX₄₋₅D), B (GX₂₋₃TX₃N), and C (GDD) (data not shown). Interestingly, domains A, B, and C were also present either in ABC or CAB orientations in several mosquito-borne viruses belonging to the recently proposed taxon *Negevirus* (Nabeshima et al. 2014).

CiLV-N (Roy et al. 2013b) and CiNSV (Cruz-Jaramillo et al. 2014) share a similar genome structure with *Orchid fleck virus* (OFV), the type member of the very recently proposed genus *Dichorhavirus* (Dietzgen et al. 2014) (Fig. 6). The analyses of amino acid similarities (Tables 4 and 5) established a close phylogenetic relationship among CiLV-N, CiNSV, and OFV (Roy et al. 2015). However, information on infection of citrus by OFV or infection of orchids by CiLV-N or CiNSV in nature is not currently available. The partial nucleocapsid gene (N) of 25 CiLV-N infected tissues from Meta and Casanare states of Colombia and Querétaro

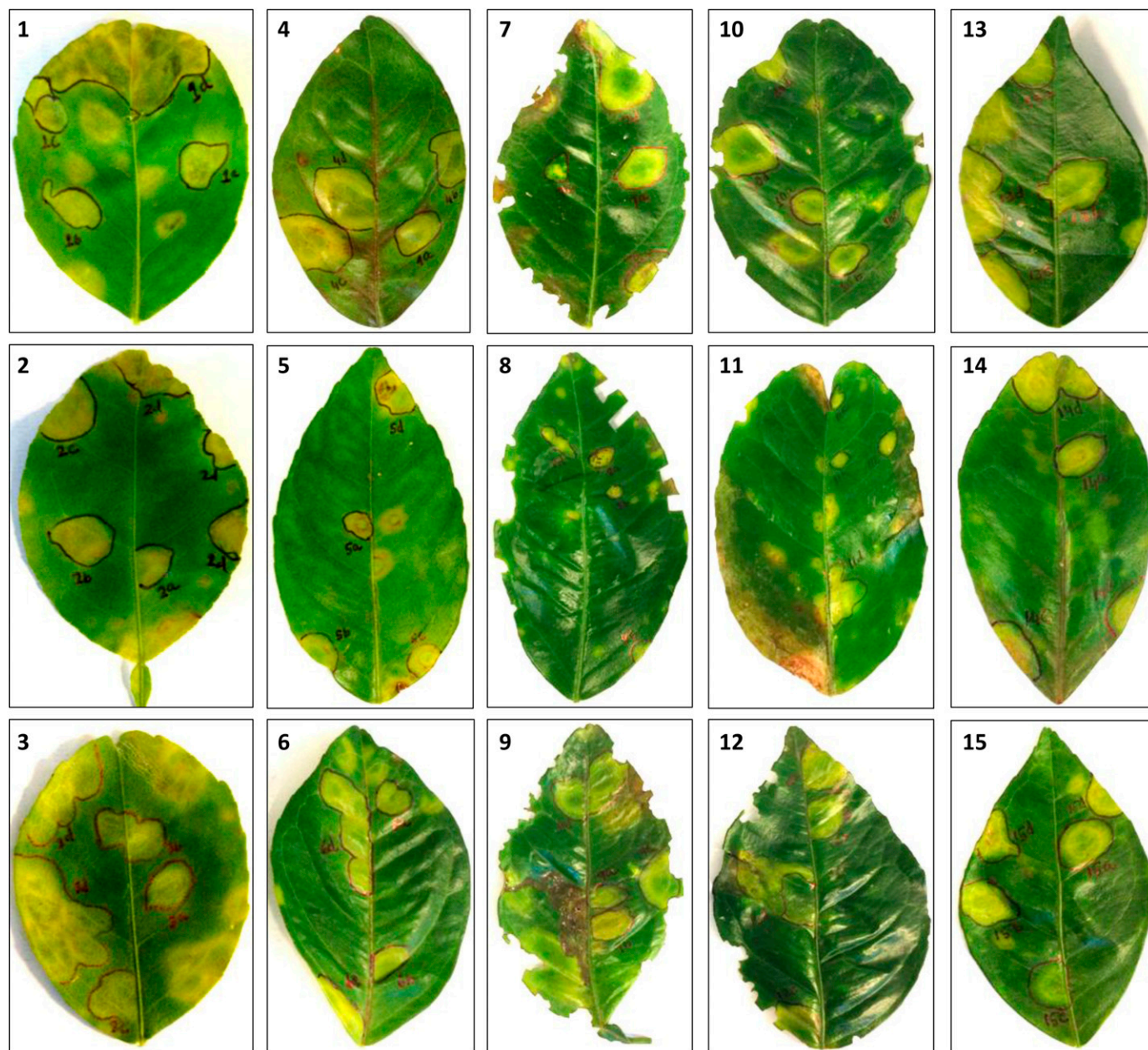


Fig. 4. Detection of cytoplasmic and nuclear citrus leprosis viruses in mixed infection from single and multiple lesions collected from individual field-collected leaves and after successful experimental mite transmission. We choose three leaves from each leprosis-infected citrus plant infected either naturally (4 to 6, 7 to 9, 10 to 12, and 13 to 15) or experimentally (1 to 3). We marked individual lesions and tested for presence of leprosis viruses using leprosis species specific primers by reverse transcription-polymerase chain reaction.

state of Mexico were amplified and sequenced. Alignments of the N gene sequences shared 80 to 99% sequence identity with the previously reported CiLV-N and CiNSV isolates and formed two separate clusters in the dendrogram (data not shown), consistent with the idea that these viruses are somewhat distinct from each other and from OFV.

Virus-mite relationships. All three of the major citrus leprosis viruses (CiLV-C, -C2, and -N) have been reported in the Orinoco region of Colombia. During the survey in 2014, citrus leaves with leprosis symptoms as well as possibly viruliferous mites were collected from citrus groves in Casanare and Meta states, and the virus infection status of both plants and mites was assessed. Both single and multiple leaf lesions collected from leprosis-infected citrus groves were infected with both CiLV-C2 and CiLV-N (Fig. 4). RT-PCR using RNA from batched potentially viruliferous field collected mites also identified CiLV-C2 and CiLV-N in the mites. Surprisingly, CiLV-C, which is less prevalent in this region, was detected in the field collected mites. This is the first record of detection of all three leprosis viruses in *Brevipalpus* mites collected

from the same area. In another experiment, viruliferous field-collected mites (confirmed by RT-PCR) were fed on leaves of healthy sweet orange seedlings. Virus-free *Brevipalpus* mites were also fed on field plants infected with CiLV-C2 and CiLV-N, and then used to inoculate healthy sweet orange seedlings. Four to six weeks after mite transfer, leprosis symptoms developed on healthy sweet orange leaves (Fig. 4). Small circular chlorotic spots were observed initially and over time each spot increased in size and coalesced into large chlorotic areas 110 to 120 days after the inoculation access period (IAP). Interestingly, both inoculation treatments generated mixed infections of CiLV-C2 and CiLV-N. None of the leprosis symptomatic plants became positive for CiLV-C.

In mite surveys, *B. californicus* s.l. associated with citrus in Mexico was found mainly at higher elevations, between 1,500 and 2,200 m above sea level (masl), in the central Mexican plateau, and was the only *Brevipalpus* species present at these altitudes (Fig. 7A and G). However, *B. californicus* s.l. was also collected in Tamaulipas State, at about 150 masl. Salinas-Vargas et al. (2013) identified *B. californicus* s.l. on citrus, coexisting with *B. phoenicis* s.l.,

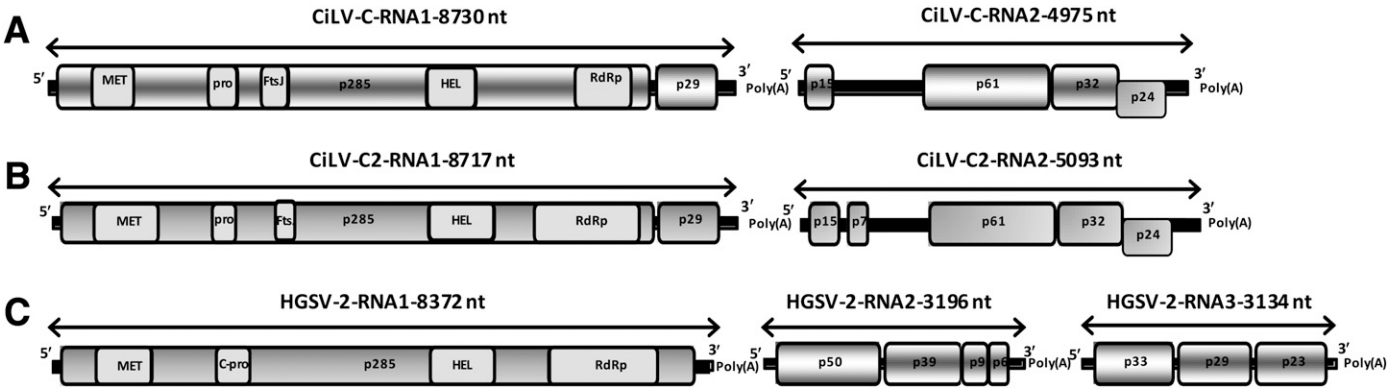


Fig. 5. Schematic genome organization of cytoplasmic viruses associated with citrus leprosis disease. **A**, *Citrus leprosis virus C* (CiLV-C), **B**, CiLV-C2 (genus *Cilevirus*), and **C**, *Hibiscus green spot virus 2* (HGSV-2) (genus *Higrevirus*).

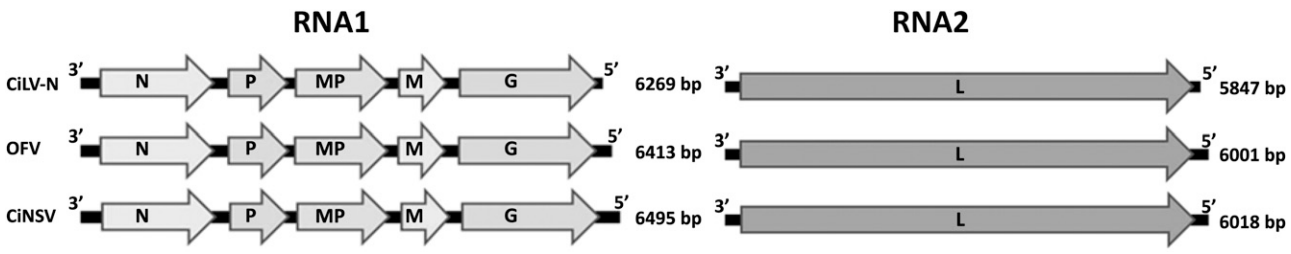


Fig. 6. Genome organization of nuclear *Citrus leprosis virus* (CiLV-N), *Citrus necrotic spot virus* (CiNSV), and *Orchid fleck virus* (OFV) members of the newly proposed genus *Dichorhavirus*. The relative sizes of individual viral genes are shown and the genome is encoded in the negative-sense orientation. Genes N, P, MP, M, G, and L encode nucleocapsid-, phospho-, movement-, matrix-, glyco-, and long polymerase proteins, respectively.

TABLE 4. Percent nucleotide (above diagonal *) and amino acid (below diagonal *) identities of RNA1 genomes of *Citrus leprosis virus N* (CiLV-N), *Citrus necrotic spot virus* (CiNSV), and *Orchid fleck virus* (OFV) associated with leprosis-like symptoms^a

SI number	Viruses	RNA1																	
		ORF1 (p49)			ORF2 (p27)			ORF3 (p42)			ORF4 (p20)			ORF5 (p63)			Complete genome		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1	CiLV-N	*	97	90	*	96	92-93	*	96	90	*	97	89-90	*	96	88	*	93	88
2	CiNSV	98	*	90	97	*	93	95	*	90	96	*	90	96	*	88	ND	*	88
3	OFV	97-98	97-98	*	97	97	*	93	93	*	93-94	97	*	92	92	*	ND	ND	*

^a ND indicates data not determined.

at several locations on the Yucatán Peninsula, at less than 100 masl, and De Leon (1961) reported *B. californicus* s.l. associated with citrus at about 450 masl in Morelos, Nuevo Leon State, Mexico. In contrast, *B. yothersi* was prevalent in lowlands (less than 100 masl), throughout both the Pacific and the Atlantic Coastal plains, although this species was also collected in Morelos state, at about 1,000 masl (Fig. 7B and G). De Leon (1961) identified *B. phoenicis* s.l. in several Mexican lowland localities. Later Izquierdo Castillo et al. (2011) also identified *B. phoenicis* s.l. mites as the only *Brevipalpus* species found associated with leprosis in the lowlands of Tabasco and Chiapas states.

Our study of this material, utilizing multiple distinguishing characters (Table 3) including cuticular microplates (Fig. 7D to E), indicates that the Mexican species previously identified as *B. phoenicis* s.l. (i.e., a member of the *B. phoenicis* species group) is actually *B. yothersi*. The true *B. phoenicis* sensu stricto, while recorded on *Citrus* spp. hosts, has not yet been associated with citrus leprosis (Beard et al. 2015). Collections of *Brevipalpus* mites from Colombia, Brazil, and Mexico found two different *Brevipalpus* species, in two different species groups, associated with citrus leprosis, *B. yothersi* (*B. phoenicis* species group: tarsus II with two solenidia; dorsal seta *f2* absent) and *B. californicus* s.l. (*B. californicus* species group: tarsus II with two solenidia; dorsal seta *f2* present). Here, the species collected in association with the cytoplasmic type of citrus leprosis virus from Colombia, Brazil, Mexico, and Panama (G. Leon, J. Mineiro, G. Otero-Colina, and R. H. Brlansky, *personal observations*) has been identified as *B. yothersi*, a former synonym of *B. phoenicis* (Geijskes) (Fig. 7A). *B. obovatus* was not found in these surveys. Together with elevation data, this indicates that *B. californicus* s.l. thrives mostly in temperate places when associated with citrus, while *B. yothersi* prefers warmer localities. Either species may be present at intermediate altitudes. In Mexico, nuclear viruses are most prevalent at high altitudes or in temperate localities, associated with *B. californicus* s.l., and cytoplasmic viruses are most prevalent in warm lowland regions, where *B. yothersi* is the most abundant species.

Phylogenetic analyses. Phylogenetic analyses of the RdRp2 domain and the p24 protein of cileviruses, several insect viruses and several insect-transmitted plant viruses were constructed with the neighbor joining method. The RdRp2 domain, deduced from the amino acid sequence of the replicase gene, is present in a diverse set of plant and insect viruses. The RdRp phylogeny included several clades and overall the bootstrap values within each of the clades (*Cilevirus*, *Negevirus*, and *Blunervirus*) were very strong, ranging from 86 to 100 (Fig. 8A). Negevirus formed a sister clade with cileviruses, including CiLV-C, but with a bootstrap value of only 70, consistent with unknown common ancestors. Interestingly, within the cilevirus clade, CiLV-C2 shared a common ancestor with the hibiscus-infecting cilevirus, and along with *Linguistrium ringspot virus* formed a sister clade with CiLV-C, with a bootstrap value of 100. A similar tree topology was displayed when the helicase domain protein sequences were aligned and compared (data not shown).

The p24 genes of the cilevirus group are novel, and many plant viruses do not have this type of p24 gene (Kuchibhatla et al. 2014). In addition, some other groups did not annotate homologous

sequences as p24 genes. Therefore only a handful of p24 proteins of this type are annotated in GenBank and other publically available databases. Advanced homology detection methods such as HHblits and HHpred (Kuchibhatla et al. 2014) were utilized to identify p24 genes in virus sequences available in the GenBank, including remote homologs of the p24 gene of the cilevirus group (Blastp e-04 to -31). Examination of the p24 tree showed that the genera *Cilevirus* and *Higrevirus* were well separated from the mosquito viruses. The speciation event that separated the cilevirus and higrevirus groups from the blunervirus and negevirus groups had a strong bootstrap value of 92 (Fig. 8B).

CiLV-N and CiNSV shared a very similar overall genome structure with OFV, the type member of the proposed genus *Dichorhavirus*, as well as with the recently sequenced *Coffea ring spot virus* (Ramalho et al. 2014). Nucleocapsid (N), glycoprotein (G), and long polymerase (L) genes from these genomes, conserved among all the members of the family *Rhabdoviridae*, had the greatest regions of similarity irrespective of plant hosts. Phylogenetic analysis of the N, G, and L genes calculated with the neighbor-joining method clearly showed that CiLV-N and CiNSV were distinct members of the genus *Dichorhavirus*, as they were found on separate branches from that occupied by OFV (data not shown). In contrast, an analysis of a phylogenetic tree calculated from the same sequence data with the maximum parsimony method was consistent with the interpretation that CiLV-N and CiNSV were citrus strains of OFV rather than a distinct virus (data not shown). Final taxonomic classification of CiLV-N and CiNSV will remain unclear until sequence data from more CiLV-N and CiNSV isolates are available.

Replication of CiLV-C, CiLV-C2, and CiLV-N inside mites. Based on the identifications of mites from leprosis-affected areas in Colombia and Mexico, the two most important species of the genus *Brevipalpus* for CiLV transmission, *B. yothersi* and *B. californicus* s.l., were assessed for the replication of CiLV-C, -C2, and -N. The Orinoco region of Colombia is the only place where all three types of citrus leprosis viral pathogens are known to be present. To determine whether CiLV-C, -C2, and -N replicate inside *Brevipalpus* spp., an RT-PCR assay specific for the replicative strand of viral RNA was designed to detect the presence of the positive-sense (for CiLV-N) or negative-sense (CiLV-C and -C2) RNA strand inside the mite. RNA was extracted from *Brevipalpus* mites collected from leprosis-infected citrus groves in Meta, Colombia, and the same RNA templates were utilized to make cDNA with leprosis virus species specific replicative strand primers.

A positive-sense strand specific PCR amplicon specific for CiLV-C and CiLV-C2 and a negative-sense strand specific PCR amplicon specific for CiLV-N were obtained from potentially viruliferous mites, confirming the presence of cytoplasmic and nuclear leprosis virions in the mites. In parallel, negative and positive strand specific PCR for cytoplasmic (CiLV-C and -C2) and nuclear (CiLV-N) leprosis viruses, respectively, generated replicative strand specific amplicons for CiLV-C, CiLV-C2, and CiLV-N from the same RNA template, confirming the presence of replication intermediates inside the mites. Both positive and negative strand specific RT-PCR assays performed without prior RT failed to produce any amplicons (Fig. 9).

DISCUSSION

Presently, five well-defined viruses are associated with citrus leprosis syndrome in the Americas. Three of them, CiLV-C (Locali-Fabris et al. 2006; Pascon et al. 2006), CiLV-C2 (Roy et al. 2013a), and HGSV-2 (Melzer et al. 2012), produce particles primarily in the cytoplasm of infected cells, and the remaining two viruses CiLV-N (Roy et al. 2013b) and CiNSV (Cruz-Jaramillo et al. 2014) produce particles primarily in the nucleus of infected cells. The

TABLE 5. Percent nucleotide (above diagonal *) and amino acid (below diagonal *) identities of RNA2 genomes of *Citrus leprosis virus* N (CiLV-N), *Citrus necrotic spot virus* (CiNSV), and *Orchid fleck virus* (OFV) associated with leprosis-like symptoms^a

SI number	Viruses	RNA2			Complete genome		
		ORF1 (p212)					
		1	2	3	1	2	3
1	CiLV-N	*	96	89–90	*	93	87–88
2	CiNSV	97	*	88–89	ND	*	88–89
3	OFV	97	95–96	*	ND	ND	*

^a ND indicates data not determined.

viruses associated with leprosis are clearly an emerging issue, as the distribution within the new world seems to be expanding rapidly (Fig. 1).

The plant host situation for leprosis-related viruses is rather unique. No systemic plant hosts have been identified for any of the viruses associated with citrus leprosis. Very recently, in an experimental host range study for CiLV-C, 140 species from 43 families were

tested (Garita et al. 2014), and 40 species belonging to 18 families produced either localized chlorotic and/or necrotic lesions on leaves and were PCR-positive for CiLV-C. *Dieffenbachia* sp. had never been considered as experimental host for CiLV-C, but survey work in Colombia demonstrated that CiLV-C2 infects *Dieffenbachia* sp., *S. glutinosa*, and *H. rosa-sinensis* (Fig. 2). RT-PCR was used to determine that sweet orange, *Dieffenbachia* sp., and *S. glutinosa* in

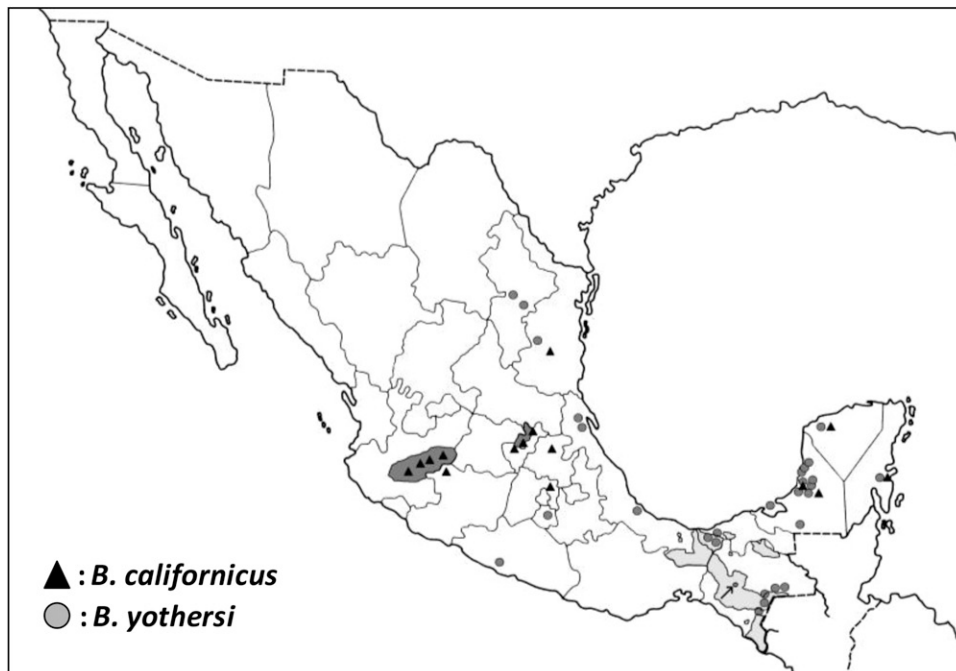
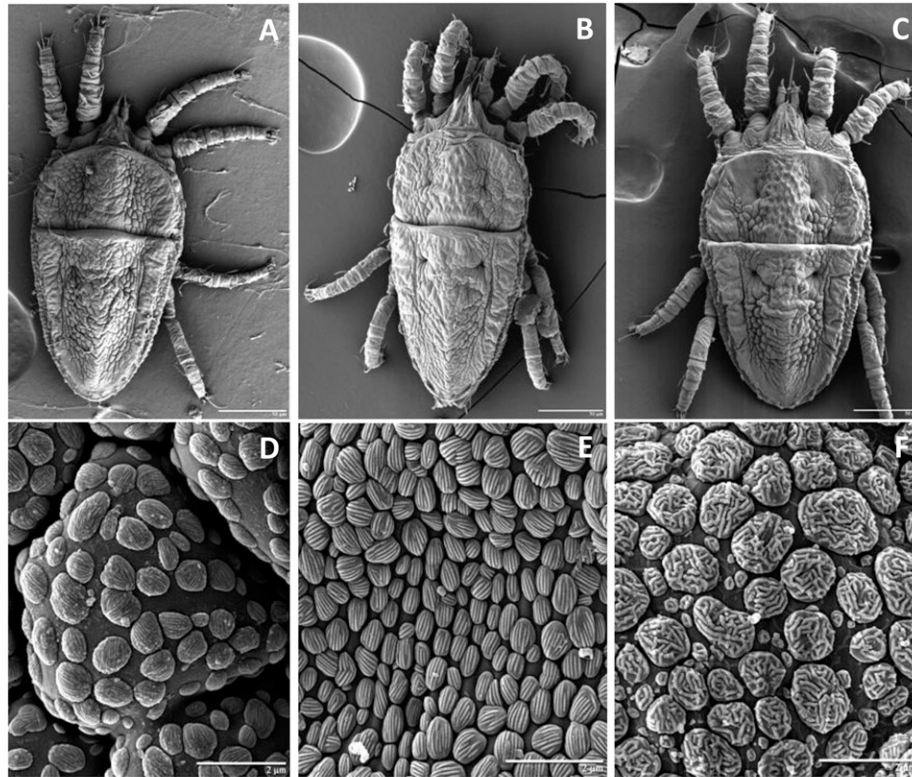


Fig. 7. Morphological identification of *Brevipalpus* species associated with *Citrus leprosis virus C* (CiLV-C) and CiLV-N using low temperature scanning electron microscopy. Habitus images of **A**, *B. californicus* sensu lato (s.l.), **B**, *B. yothersi*, **C**, *B. phoenicis* sensu strict (s.s.). Pattern of cuticular microplates of **D**, *B. californicus* s.l., **E**, *B. yothersi*, and **F**, *B. phoenicis* s.s. While *B. phoenicis* s.s. has been recorded from different *Citrus* spp., it has never been associated with citrus leprosis, and is included here only to indicate the differences between it and its former synonym *B. yothersi*. Bottom image shows the distribution of both *Brevipalpus* species in leprosis-free and -affected areas in Mexico. Regions in light and dark gray colors represent the presence of cytoplasmic and nuclear type, respectively. The arrow in light gray area indicates the presence of nuclear type in the state of Chiapas, Mexico.

Colombia were infected by CiLV-C2 and CiLV-N in mixed infections. This is the first report of natural infection of leprosis viruses on *Dieffenbachia*. Examination of individual lesions indicated that both viruses were frequently found in the same lesion, suggesting that both viruses may co-occur in individual mites without interfering with each

other during infection. The amplified product from *H. rosa-sinensis* had a 99% sequence identity with CiLV-C2. *H. rosa-sinensis* has also been reported as experimental host for CiLV-C (Nunes et al. 2012b).

The previously dominant leprosis species, CiLV-C (Leon et al. 2006a, b), was not detected in Meta and Casanare, Colombia

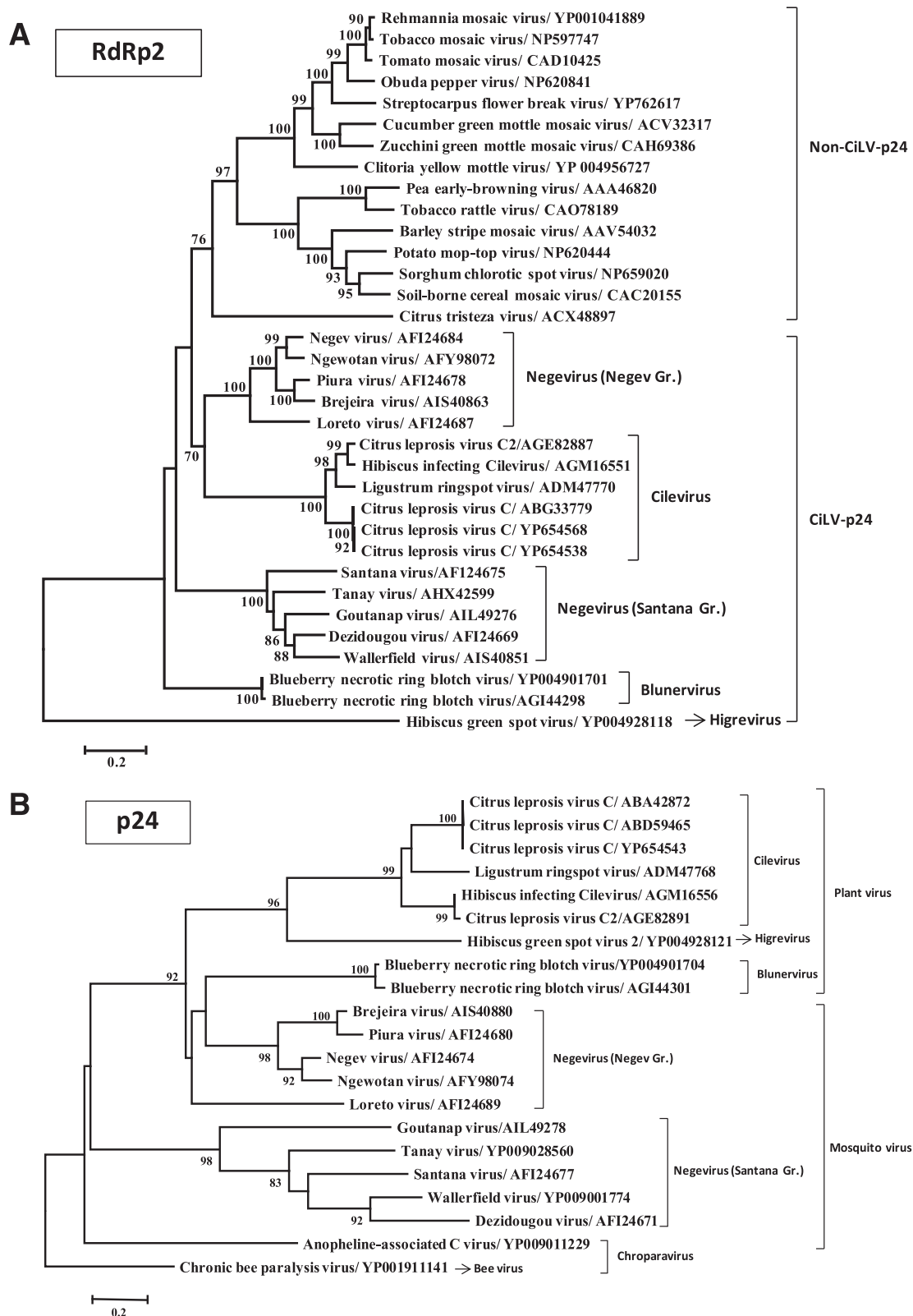


Fig. 8. Phylogenies produced using neighbor-joining methods based on genome sequence of leprosis-associated viruses with related plant and insect viruses. **A**, RNA dependent RNA polymerase 2 (RdRp2) from RNA1, and **B**, with probable internal membrane protein of the virion (p24) from RNA2 or RNA3. Phylogenies were supported by 1,000 bootstrap replicates and bootstrap values greater than 70 are shown at the nodes.

(Roy et al. 2013a). Surprisingly, CiLV-C, -C2, and -N were all detected inside the viruliferous mites collected from the Orinoco region of Colombia. Mite transmission experiments conducted using healthy mites and mixed infections of CiLV-C2 and -N viruses as a source of inoculum were successful for CiLV-C2 and -N. In parallel experiments using field-collected viruliferous mites positive for CiLV-C, -C2, and -N, the mites transmitted CiLV-C2 and CiLV-N but failed to transmit CiLV-C to healthy seedlings. This suggests that the previously predominant leprosis species CiLV-C in Colombia may be replaced by the presently dominant leprosis species CiLV-C2, perhaps due to selective advantage or preference conferred during the mite transmission to plants (Roy et al. 2013a). In an effort to find CiLV-C in Colombia, some municipalities of Tolima, Cundinamarca, and Vichada States in Colombia were also surveyed during 2013 and 2014. Suspected leprosis lesions found in *C. sinensis* and *S. glutinosa* in Tolima and Cundinamarca States and in *C. sinensis* in the State of Vichada tested positive for CiLV-C2 and CiLV-N (Fig. 1C). The absence of CiLV-C in these locations further suggested that CiLV-C is being replaced by CiLV-C2. However, single mite acquisition tests, currently limited by the sensitivity of RT-PCR assays, will be needed to test the hypothesis that mite acquisition is related to the replacement of CiLV-C by CiLV-C2.

Previously, HGSV-2 had only been reported in *C. volkameriana* and *H. arnottianus*, two species of limited agricultural importance. *C. volkameriana*'s primary importance to the citrus industry is as a rootstock, and therefore its susceptibility to the nonsystemic HGSV-2 in leaf and twig tissue is not a concern. Field surveys in Hawaii demonstrated that the host range of HGSV-2 includes mandarin and navel orange, as well as a common landscape tree, *H. tiliaceus* (Fig. 3). However, even though nearly 100% of the mandarin and navel orange trees displayed symptoms, the adjacent Persian lime trees were asymptomatic and virus-free.

Less is known about the host range of CiLV-N. CiLV-N was reported from Querétaro state, Mexico in 2013 and leprosis symptoms were observed on seven naturally infected citrus species in that state (grapefruit, lime, lemon, mandarin, sour orange, sweet lime, and sweet orange) (Roy et al. 2015). Similarly, CiNSV was also reported from grapefruit, sour orange, sweet orange and tangerine in Jalisco, Mexico (Cruz-Jaramillo et al. 2014). To determine the distribution and genetic variability among CiLV-N isolates a survey was conducted in June 2014. Apart from the seven

previously reported citrus hosts for CiLV-N, Persian lime also displayed distinct necrotic rings surrounded by yellow haloes (Fig. 2C). RT-PCR confirmed the presence of CiLV-N, extending the agricultural host list within *Citrus*.

A careful examination of *Brevipalpus* mites from Colombia, Brazil, and Mexico found *B. yothersi* and *B. californicus* s.l. associated with citrus leprosis (Fig. 7A and B). The mite species collected in association with the cytoplasmic type of CiLV from Colombia, Brazil, Mexico, and Panama has been identified as *B. yothersi*, former synonym of *B. phoenicis* (Pritchard and Baker 1952) (Fig. 7A and B). *B. yothersi* was listed by Beard et al. (2013) as *B. phoenicis* group species *B.* However, based on a detailed examination of specimens of several species (including type material) in the *B. phoenicis* complex using LT-SEM and traditional light microscopy, *B. yothersi* was reinstated as a valid species and redescribed (Beard et al. 2015) and is considered to be a recognized vector of the citrus leprosis complex. The discovery of the presence of specific patterns of microplates (Welbourn et al. 2003) on the cuticle of *Brevipalpus* mites has proven to be critical for species separation and confirmation within this genus.

In Mexico two mite species were identified, *B. yothersi* associated with CiLV-C and *B. californicus* s.l. associated with CiLV-N. There are also two reports from Tabasco (G. Otero-Colina, *personal communication*) of CiLV-N or CiNSV in mixed infections with the prevalent CiLV-C population. As the co-occurrence of *B. yothersi* and *B. californicus* s.l. in southern Mexico has not been documented, we suggest that both cytoplasmic and nuclear leprosis viruses may be transmitted by *B. yothersi*. Based on RT-PCR results, the lower concentration of CiLV-N than CiLV-C was observed in symptomatic leprosis tissues, suggesting that *B. yothersi* may possibly acquire and transmit CiLV-C more efficiently than it does CiLV-N. Further experiments on CiLV transmission followed by detection and quantification of individual leprosis virus species using both *B. californicus* s.l. and *B. yothersi* will provide a better understanding of virus-vectors relationships.

The lack of a natural systemic plant host is quite atypical for plant viruses, raising the question of whether or not these two completely unrelated virus groups are actually insect viruses that have convergently evolved a unique approach for mite-to-mite transmission. There is evidence for a relationship between CiLV-N and mites, as electron lucent viroplasm and rod shaped particles of

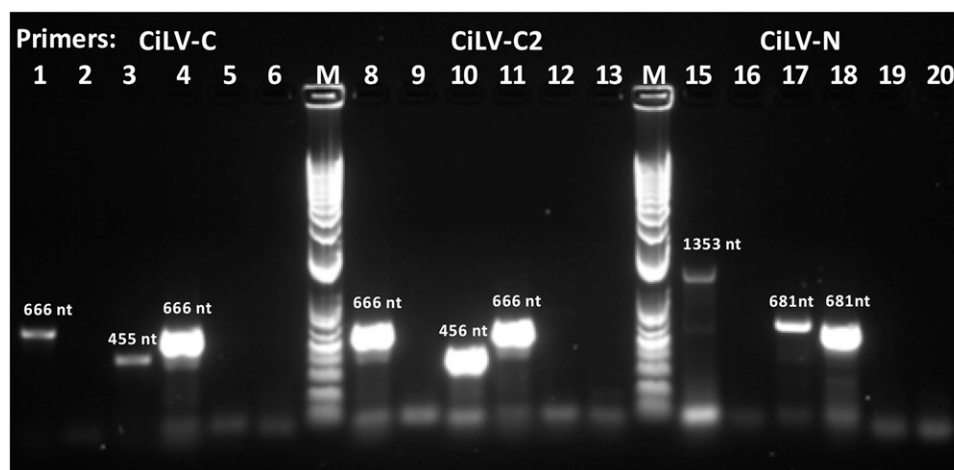


Fig. 9. Native and complementary strand specific reverse transcription-polymerase chain reaction (RT-PCR) analysis for leprosis-associated viruses (*Citrus leprosis virus*-C, -C2, and -N) with species specific primers. Positive sense CiLV-C and -C2 amplified fragment had a length of 666 bp in both cases (lanes 1 and 8), whereas negative-sense CiLV-N produced 1,353-bp amplicon (lane 15). Negative-sense CiLV-C and -C2 amplified fragment had a length of 455 and 456 bp, respectively (lanes 3 and 10), whereas positive-sense CiLV-N produced 681-bp amplicon (lane 17). A positive control PCR was performed using total RNA of CiLV-C, -C2, and -N positive isolates (lanes 4, 11, and 18). A negative control without RT step was performed for CiLV-C (lane 2), CiLV-C2 (lane 9), and -N (lane 16). Specificity of leprosis species specific primers were also checked using cDNA of CiLV-C and specific primers of CiLV-C2 and CiLV-N (lanes 4 and 5), cDNA of CiLV-C2 and specific primers of CiLV-C and CiLV-N (lanes 12 and 13), and cDNA of CiLV-N and specific primers of CiLV-C and CiLV-C2 (lanes 19 to 20). Lanes M: 1-kb plus DNA molecular weight ladder.

CiLV-N were found in cytoplasm as well as in the nucleus of the viruliferous *Brevipalpus* mites. These cytopathic effects are similar to what was observed in CiLV-N infected plant tissues, suggesting that the CiLV-N is replicating inside the mite cells (Kitajima and Alberti 2014). This is not surprising, as there are a fair number of rhabdoviruses that infect both plants and insects (Ammar et al. 2009).

The situation is much less clear for cileviruses. CiLV-C was first observed in thin sections of viruliferous *B. phoenicis* s.l. utilizing TEM (Rodrigues et al. 1997). However, Kitajima et al. (2014) suggested that the previously described image of CiLV-C in *B. phoenicis* was misinterpreted. Based on our identification criteria, we strongly suspect that the species used by Rodrigues et al. (1997) and Kitajima et al. (2014) is *B. yothersi*. Recently Kitajima and Alberti (2014) showed CiLV-C particles persistently present in mites, between membranes of adjacent cells, particularly at the basal parts of the midgut epithelium and neighbor cells. In contrast, CiLV-N particles were present in many nuclei of cells of the anterior midgut epithelium, anterior podocephalic glands, and epidermis. TEM of thin sections was unable to find intracellular viral particles and viroplasm of CiLV-C in mites, indicating that CiLV-C may not be replicating inside the mite cells.

Despite this, phylogenetic analyses suggested that there may be a relationship between cytoplasmic leprosis-associated viruses and insect viruses. The phylogeny derived from the helicase and RdRp2 domains separates the non-CiLV group of plant viruses, which lack a p24 gene, and the cilevirus group that contain a p24 gene. Overall, the independent phylogenetic tree calculated from the p24 protein sequences was very consistent with the phylogenetic trees calculated from the helicase and RdRp2 domain, with similar topologies within the cilevirus clade (Fig. 8). Taken together, *Brevipalpus* mite-transmitted or associated viruses from the genera *Cilevirus*, *Higrevirus*, and *Blunervirus* are more closely related to the insect viruses than to other plant viruses. The tree calculated from the RdRp2 domain shows a clear separation of the plant and insect viruses, but with the cilevirus clade clustering within the insect virus group.

The p24 proteins of cileviruses, higrevirus, and blunervirus have four transmembrane domains and this class of p24 proteins probably acts as a major structural component of the virion (Kuchibhatla et al. 2014). The members of the genera *Cilevirus* and *Higrevirus* produce bacilliform particles, while members of the genus *Negevirus* produce spherical particles, and the viruses were separated in the p24 tree into sister clades consistent with this particle morphology. In addition, the CiLV-C and CiLV-C2 have a p24 protein that is related to the p24 protein from the insect viruses. This suggests that the cilevirus group may have originated in the mite, and has secondarily adapted to the plant host, perhaps conferring the role of vector on the plant in this unusual plant virus system. Data consistent with virus replication in mites were obtained for CiLV-C and CiLV-C2 as well as CiLV-N. Negative strand RNAs for the positive sense viruses (CiLV-C and -C2) and positive strand RNAs for negative sense viruses (CiLV-N), known to function as replication intermediates, were detected inside *B. yothersi*. Additionally, examination of single lesions simultaneously infected with both virus types suggest that individual mites may be co-infected with multiple leprosis-related viruses, because it is assumed that the single lesion is derived from a single feeding mite, although it is not clear whether these viruses infect and utilize the same tissues within the mite. The effect of leprosis-related virus coexistence in single mites in terms of synergy, transmissibility, symptoms, virus replication, and life expectancy of *Brevipalpus* species remains unclear. Regardless, these viruses seem to have convergently evolved a unique approach for mite-to-mite transmission that warrants further study.

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